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Ranking the Factors Impacting Immunosorbent Performance

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INTRODUCTION

There are increased market pressures on the health care industry to provide protein-based therapeutics in ample amounts at reduced cost [1,2]. Needs for increases in process efficiency include that of pre-purification steps and affinity adsorption processes [1]. General inefficiencies in current adsorption processes lead to high buffer usage, buffer exchange steps, and slow volumetric throughput, thus leading to multiple steps involving volume reduction.

Affinity and ion exchange adsorption technology are scaleable technologies important to protein purification [1,3–7]. Purification methodologies based on ion-exchange or adsorption serve as excellent pre-purification steps but fail to resolve complex protein mixtures to yield a single purified protein product. Purification techniques based on affinity interactions between molecules (i.e. immunoaffinity chromatography) have rapidly evolved using a variety of biological and synthetic ligands. Immunoaffinity chromatography is a process in which the binding affinity of an antigen to a parent antibody is utilized as a basis of separation. The use of monoclonal antibodies (Mab), which display a high degree of avidity for a specific binding site (epitope) on a molecule (antigen and hapten), has greatly impacted immunoaffinity chromatography. Due to the customized avidity and specificity, monoclonal antibodies have become indispensable for both protein characterization and purification.

The high costs involved with the production of monoclonal antibodies makes immunoaffinity chromatography a cost-intensive purification technique, both at laboratory and industrial scale [4]. Furthermore, large-scale applications of immunoaffinity chromatography have been limited by the low functional efficiency that results when antibodies have been covalently anchored to supports [6]. Increasing the immunosorbent efficiency would reduce antibodies and support matrix costs. It is estimated that buffer costs contribute about 40% of the processing cost in large-scale chromatographic purifications. Lowering the operating cost of irnmunosorbents with higher functional efficiency of immobilized Mab would also reduce buffer costs by decreasing column-wash and product eluate volumes.

The performance of an immunosorbent is dependent on physical characteristics of the matrix, localized density of the immobilized antibody, and "orientation" of the antibody [5]. The chemical structure of the antibody such as lysyl-residue and carbohydrate content will also impact the above phenomena. Although immunosorbent performance is characterized on a volume or bead-averaged basis, each of these phenomena may be a function of antibody position within the bead. From a localized view of the bead or support interior, it is necessary for the immobilized antibody to be in the right "orientation" to specifically interact with the antigen in solution. Furthermore, antibody density, which is a strong function of position, can adversely impact immunosorbent efficiency. A detailed review of the literature is provided elsewhere [5,7,8]. Multi-point attachment via abun-

dant and proximal amine groups on Mabs to the support matrix will also negatively impact immunosorbent efficiency [9].

Only recently has understanding of the relationship between local site density and adsorption (affinity) site functionality, and matrix average capacity at a given matrix average site density been improved [7]. In summary, we have demonstrated the feasibility of achieving greater capacities in affinity adsorption matrices by an engineering site installation and protecting the character of the Mab. Optimization of the immunosorbent performance requires an understanding of the factors that affect the activity of immobilized antibodies. The aim of this research is to recognize and rank the factors which affect the functional efficiency of immobilized antibodies and implement techniques to alleviate these deleterious factors. We have also used pepsin digestion of immunosorbents to quantitatively correlate the increases in antigen-binding efficiencies or immunosorbent efficiency [$\eta_{(Ag)}$] to the accessibility of Fab domains. Thus, a rational design of immobilization procedures to yield a highly active immunosorbent should then be possible using the results of this study.

MATERIALS

Murine pH dependent anti-human Protein C (hPC) monoclonal antibody (8861-Mab) was provided by American Red Cross (Rockville, MD). Rabbit antisera against human Protein C, affinity-purified goat anti-mouse (whole molecule) and anti-goat/anti-mouse immunoglobulins conjugated to horseradish peroxidase (HRP), were purchased from Sigma Chemical Co. (St. Louis, MO). Goat anti-human Protein C antisera was purchased from American Diagnostics Inc. (Greenwich, CT). Emphaze™ biosupport medium was provided by 3M (Minneapolis, MN) and Affiprep™ polymeric support was purchased from Bio-Rad Laboratories (Anaheim, CA). Cellulose beads were manufactured according to the procedure outlined in [10]. Immulon II microtiter plates were purchased from Fisher Scientific. Human protein C (hPC) was provided by the American Red Cross (Washington, DC). Recombinant hPC (rhPC) was isolated from transgenic porcine whey using immunoaffinity chromatography [11]. O-phenylenediamine-2HCl tablets were purchased from Abbott Laboratories (Chicago, IL). All other reagents were purchased from Sigma at the best grade available. Immunoaffinity separations were performed with Pharmacia C-10 columns (15 cm x 1 cm), a Masterflex peristaltic pump, and a Knauer spectrophotometer. A Rainin data acquisition system was used to monitor chromatography. Columns were kept at 4°C with a Lauda Super RMT water cooler.

Description of Supports Studied

The supports used in this study were subjected to different activation chemistries and were purchased from commercial sources except for cellulose beads, which were synthesized and modified in the laboratory. The reactive end group on Affiprep was an ester moiety that reacted with the $-NH_2$ group on the proteins to yield stable amide linkages [12]. The reactive end group on Emphaze was an azlactone that reacted with nucleophiles (ex: $-NH_2$ moieties on the antibody/proteins) to yield stable covalcnt linkages [13]. Cellulose beads 700–900 pm in diameter were prepared and characterized according to the procedure detailed elsewhere [10]. Cellulose beads having a solids content of 3.5% (wt/wt) were chosen for this study. Cellulose beads were derivatized according to the procedure outlined elsewhere [14] to yield reactive cyano-end groups. Briefly, 10 ml of underivatized beads were placed on a coarse sintered funnel and washed first with water, then acetone:water (30:70), and finally with acetone:water (60:40). The beads were drained by mild suction and transferred to a 50-ml beaker, suspended in 10 ml of acetone:water (60:40) and cooled to 0°C. 750 µl of a 0.1 mg/ml stock solution of 1-cyano-4-(dimethylamino)-pyridinium-tetraflurborate (CDAP) in acetonitrile was then added to the reaction flask and the contents were stirred gently for one minute. 600 µl of a 0.2 M aqueous solution of tricthanolamine (TEA) was then added dropwise and the reaction was allowed to proceed for another two to three minutes. Upon completion of the activation step, the contents of the flask were emptied into a beaker containing 200 ml of ice-cold 0.05 N HCl and the beads were allowed to settle for 15 minutes. The activated beads were washed multiple times with ice-cold water and were immediately used for coupling.

METHODS

Synthesis of Modified Mab and Modified rhPC

The reactive amino moieties on 8861-Mab and rhPC were covalently modified using an acetic acid ester of N-hydroxy succinimide [15]. [Note: 8861-Mab will be referred to as the Mab in the remainder of the paper]. A 3 mg/ml solution of Mab or rhPC in borate buffer (0.05 M borate, 0.14 M NaCl, pH 8.6) was slowly mixed with 10 µl of 200 molar excess of ester in di-methyl-formamide. The pH of the reaction was maintained at 8.5 by the addition of 1 N NaOH and the reaction was allowed to proceed for 24 hours at 4°C. The unreacted ester was quenched by adding 40 molar excess of glycine using a 0.4 M glycine stock solution at pH 8.5. After four hours of stirring, the modified Mab or the modified

dant and proximal amine groups on Mabs to the support matrix will also negatively impact immunosorbent efficiency [9].

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immunosorbents prepared using Emphaze biosupport medium, 125 mgs Emphaze beads were suspended in 1.0 ml coupling buffer containing 1.0 to 1.2 mgs of Mab to yield 1.0 ml of affinity sorbent [13]. Upon the completion of the immobilization step, blocking and washing steps similar to those described above were performed. For immunosorbents prepared using Affiprep. Affiprep was first washed with 10 mM sodium acetate buffer, pH 4.5 and was suspended as a 50% (vol/vol) slurry in the coupling buffer, 0.1 M MOPS, 0.15 M NaCl at pH 7.2 (MOPS) [12]. The supernatant was pipetted from the top of the resin and 1.0 to 2.0 mg/ml Mab in MOPS added to the tubes and the slurry was rotated overnight at 4°C. Upon completion of the coupling reaction, the gel was allowed to settle and the supernatant was pipetted and saved for ELISA assays. The residual active sites on the resin were blocked with 1.0 M ethanolamine for 1 hour at 4°C, upon which the supernatant was drawn and saved for ELISA assays. The gel was washed in a column mode with 0.05 M Tris, 0.1 M NaCl, pH 7.0 until the absorbance of the column effluent dropped to zero. The columns were stored in the ligand coupling buffer at 4°C. For immunosorbents prepared using cyano-activated cellulose beads, 1–2 ml of activated beads were washed briefly with ice-cold water followed by ice-cold MOPS. The MOPS buffer was drained and a 1–2 mg/ml of Mab solution in MOPS was added to the beads and the coupling reaction was allowed to proceed at 4°C for 24 hours. Upon completion of the coupling reaction, the beads were allowed to settle and the post-coupling treatment as described earlier was carried out.

TABLE I Effect of rhPC-FMA on Mab coupling efficiency FMA-masked and unmasked Mab were immobilized on both Affiprep, Emphaze, and cellulose beads, as described in the Methods section. Mab coupling efficiency was determined as a ratio of total Mab bound to total Mab challenge. Coupling efficiency obtained for unmasked Mab was assigned a maximum value of 100% and coupling efficiencies obtained with masked Mab are reported as a percent fraction of the maximum

Ratio of FMA[a] /Mab	*% Coupling Efficiency (Normalized)*	*Mab density[b]*
Affiprep™		
0/1	100 ± 7 %	0.4
6/1	85 ± 4 %	0.4
EMPHAZE AB1		
0/1	100 ± 4 %	1.0
6/1	55 ± 4%	1.1
Cellulose Beads		
0/1	100 ~ 4 %	0.8
2/1	88 ± 4 %	0.7
6/1	63 ± 4 %	0.5

a. Lysyl modified rhPC used as FMA.
b. Milligram (mg) Mab per ml of gel.

Preparation of "FMA-masked" Immunosorbent

Immobilizations of Mab in the presence of FMAs were carried out according to the procedure outlined elsewhere [8]. Briefly, Mab was incubated with the modified rhPC (FMA) at a ratio of 1:2 or 1:6 (as indicated in the legends) and the FMA:Mab complex was then incubated with the activated support matrix. Upon completion of the coupling, the unbound FMA:Mab complex was washed and the reversibly bound FMA was eluted in a column-mode using 2M NaSCN.

Mab Determination by ELISA

The amounts of Mab in feed and coupling step supernatants and washes were determined by the ELISA procedure detailed elsewhere [7,8]. In brief, microtiter plates coated with anti-mouse IgG (whole molecule) were used to immunocapture Mab from various chromatographic fractions and the bound Mab was detected with rabbit anti-mouse-horseradish peroxidase (HRP) conjugate. The bound chromophore was detected at 490 nm. The total Mab bound to the support was determined by a difference between the total Mab in the coupling solution and the total Mab in the coupling step supernatants and subsequent washes.

Immunoaffinity Chromatography

Analyses of all immunosorbents were performed in a column mode as detailed elsewhere [7,8]. Columns packed with approximately 1–2 ml of immunosorbent were cleaned with at least 10 column volumes (CV) of 2 M NaSCN, and then equilibrated with at least 20 CV of loading buffer (20 mM sodium citrate, 80 mM sodium chloride, pH 6.5). After equilibration, 95% pure rhPC (isolated from transgenic pig whey) at a concentration of 1–2 mg/ml was loaded at about 1 ml/min until the breakthrough front leveled off. The columns were then washed with the loading buffer until the baseline was reached. Bound rhPC was eluted with a two step sequence of a pH 10 buffer (0.1 M sodium bicarbonate, 0.15 M sodium chloride. pH 10) and then 2 M NaSCN elution. The columns were re-equilibrated in the loading buffer. All chromatographic fractions were saved for rhPC determination by ELISA. Each column was subjected to a minimum of three consecutive runs.

Determination of rhPC in Feed and Column Eluates

The amount of rhPC antigen in various chromatographic fractions was determined using the procedure outlined in [I 1]. In brief, microtiter plates coated with rabbit anti-hPC antibodies were used to immunocapture rhPC from various chro-

matographic fractions and the bound rhPC was detected with a sandwich of goat anti-hPC and HRP-conjugated sheep anti-goat antibodies at 490 nm.

Pepsin Digestion of Immobilized Mab

Immunosorbents prepared under various conditions were digested with pepsin according to the procedure detailed in [8]. In brief, a 200 µl of a 50% (vol/vol) slurry of imrnunosorbent was pipetted into screw cap tubes in replicates to yield a final immunosorbent volume of 100 p l Immunosorbents were washed with 0.1 M sodium citrate, pH 4.2 and suspended in 100 µl of citrate buffer. 10 µl of a 10 mg/ml solution of pepsin in 0.1 M acetate buffer at pH 4.5 was added to the reaction tubes and digestion was carried out at 37°C for 5 hours. The digestion was terminated with an addition of 12 µl of 1M Tris-base and the reaction mixture was centrifuged for 10 mins at 2000 x g and the supernatant was subjected to total $(Fab)_2$ determination.

Determination of $(Fab)_2$ in Pepsin Digest

The total amount of $(Fab)_2$ released in the pepsin digests was determined according to the procedure detailed in [8]. Briefly, niicrotiter plates coated with Fab specific murine antisera were used to capture $(Fab)_2$ from standard solutions and supernatants obtained from pepsin digestion. Bound $(Fab)_2$ was detected with HRP conjugated goat anti-mouse IgG at 490 nm.

RESULTS

Modification of the Reactive Primary Amines

The number of available reactive primary amines on rhPC and Mab were modified by covalent and irreversible reaction with an ester. The extent of covalent modification was determined by the ability of modified rhPC and modified Mab to react with Trinitrobenzene-sulfonic acid (TNBS). Using the procedure outlined elsewhere [16], it is estimated that 55 ± 3% of the primary amines on Mab and 60 ± 2% of the primary amines of rhPC were modified (8).

Antigen Binding Experiments

The relative binding stabilities of immunocomplexes formed in a solution from unmodified and modified rhPC and native hPC from plasma with Mab, and mod-

ified Mab with native hPC from plasma, were screened by ELISA as outlined elsewhere [8,17]. The rhPC was immunopurified to 95% purity by randomly coupled Mab [11]. Figure 1 presents the ELISA signal of immunosorbed rhPC (native or modified):Mab complex and the ELISA signal of hPC: modified Mab complex, respectively, which had been first formed in solution and then immunocaptured. In general, the ELISA signal of unmodified rhPC: and hPC:Mab and rhPC:modified Mab complexes showed similar increases with increasing antigen concentration. The ELISA profile of the modified rhPC showed a slightly higher signal than unmodified rhPC and hPC at antigen to Mab molar stoichiometries of 1/1 and 2/1. The profile was otherwise similar for modified rhPC (FMA), rhPC, and hPC with a sharp maximum at 6/1 molar ratio of antigen/Mab [8].

Effect of FMA on Immobilization of Mab

Table I gives the local Mab density obtained with immobilizations of "unmasked" and "FMA-masked" Mab on Affiprep, Emphaze. and Cellulose beads. Mab coupling efficiencies obtained with unmasked Mab were assigned a maximum value of 100%, and coupling efficiencies obtained with FMA-masked Mab were reported as a percent fraction of the maximum value. This is referred to as the percent coupling efficiency (normalized). A 12% reduction in coupling efficiency was observed for FMA-masked Mab, at a ratio of 2:1 (FMA/Mab) on cellulose beads. A similar (10–15%) reduction in coupling efficiencies was also observed for FMA-masked Mabs at a FMA/Mab ratio of 2:1 on Affiprep and Emphaze beads (data not shown). A 40–50% reduction in the amount of Mab immobilized was observed for FMA-masked Mab, at a ratio of 6:1 (FMA/Mab) on upon Emphaze, and cellulose beads. Similar reductions in coupling efficiencies were obtained for Mabs immobilized in the presence of low-molecular weight synthetic FMAs (data not shown).

Prior to testing the immunosorbent capacity, buffers to be used in the course of chromatography were passed through the appropriate column and all the column washes were assayed for either leached Mab or rhPC-FMA by EIA/ELISA. No detectable leakage of covalently bound rhPC (when used as FMA) or Mab, was observed in the column washes of the blank operation for Ernphaze (sensitivity of Mab ELISA assay: 0.1 ng/ml).

Analysis of Coupling Efficiencies

Table II gives the coupling efficiencies for both unmodified and modified Mab. Conventional immobilization methods via random coupling under fast reacting

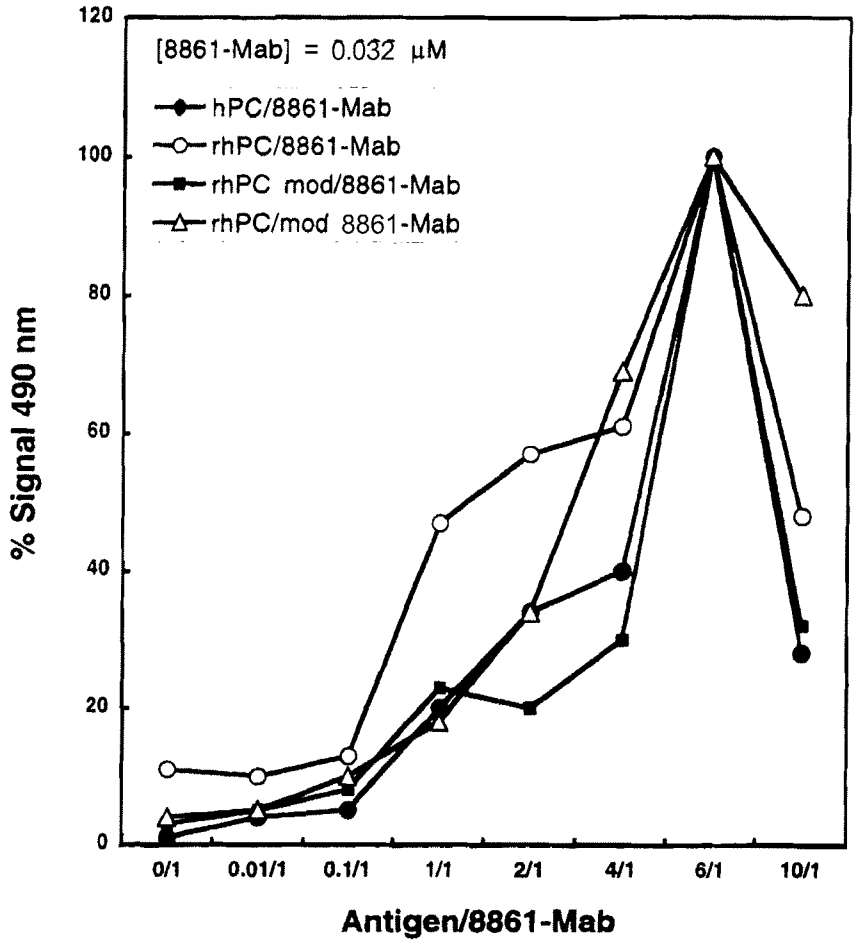

FIGURE 1 Avidity Experiments Increasing concentrations of hPC and rhPC were incubated with Mab (0.032 µM) for one hour at 25°C, and then added to rabbit anti-hPC coated wells. A typical reaction volume was 100 µl and the Mab sample was incubated with hPC or rhPC sample in a 1:1 ratio by volume. The data was averaged from triplicate applications at each indicated antigen to Mab ratio. After one hour of incubation at 37°C, goat anti-mouse IgG HRP conjugate was added to the wells and incubated for one hour. The hPC or rhPC/Mab complex was detected and quantified with OPD and read at 490 nm

conditions gave coupling efficiencies of 98–100% for a wide range of Mab loading studied. The two-step method (controlled coupling permeation followed by fast reaction) gave coupling efficiencies of 95% and 47%, giving final Mab densities of 1.1 and 9.3 mg/ml of gel, respectively.

TABLE II Mab coupling and antigen binding efficiency (η_{Ag}) of unmodified and modified Mabs under different immobilization conditions. Unmodified and modified Mab were immobilized on both Emphaze by the manufacturer's procedure, as outlined in the Methods section. Unmodified Mab was also immobilized by a 2-step method that involved controlled permcation and fast coupling (7). Mab coupling efficiency was determined as a ratio of total Mab bound to total Mab challenge. Coupling efficiency obtained for unmasked Mab was assigned a maximum value of 100% and coupling efficiencies obtained with masked Mab are reported as a percent fraction of the maximum.

Immunosorbents described in table II were challenged with pure rhPC at a concentration of 0.8–1.0 mg/ml. The capacity was determined by breakthrough analysis, as described in the Methods section and in the legend to TABLE III. Immunosorbents prepared were also subjected to pepsin digestion, as described in the Methods section. The total amount of $(Fab)_2$ released was measured by ELISA

	Coupling Method	*Coupling Efficiency*	*Actual Mab density*[a]	*Functional Efficiency* (η_{Ag})	*mg (Fab)2/mg total Mab*	*% A (Fab)2*
Native 8861-Mab	Reference	100%	0.9 mglml	23 ± 3%	0.014 mg/mg	100 ± 4%
	Reference	98%	11.8 mg/ml	26 ± 2%	0.014 mg/mg	100 ± 4%
Modified Mab	Reference	99%	1.0 mg/ml	2 0 ± 1%	0.012 mg/mg	100 ± 4%
	Reference	97%	9.2 mg/ml	31 ± 5%	0.012 mg/mg	86 ± 2%
Native Mab	2-Step	95%	1.1 mg/ml	23 ± 2%	0.013 mg/mg	92 ± 4%
	2-Step	47 %	9.3 mg/ml	58 ± 6%	0.016 mg/mg	92 ± 3%

a. mg Mab per ml of gel.

TABLE III Effect of rhPC-FMA on antigen binding efficiency (η_{Ag}) Affiprep, Emphaze, and cellulose-based immunosorbents described in TABLE I were challenged with pure rhPC at a concentration of 0.8–1.0 mg/ml. Pure rhPC was loaded until the breakthrough front leveled off. Bound antigen was eluted with 2M NaSCN. The eluate fractions were assayed for rhPC by ELISA, as described in the Methods section. Antigen binding efficiency was calculated assuming a 2:1 binding stoichiometry of antigen with antibody.

Immunosorbents prepared with unmasked and masked antibodies were subjected to pepsin digestion as described in the Methods section. The total amount of $(Fab)_2$ released was measured by ELISA. Total $(Fab)_2$and (η_{Ag}) obtained for unmasked Mab was assigned a maximum value of 100%. and (η_{Ag}) and total $(Fab)_2$ obtained with masked Mab are reported as a percent fraction of the maximum

Ratio of FMA[a] /Mab	*% Efficiency*	*% A Efficiency*	*mg (Fab)2 released / mg total Mab*	*% ΔFab)2*
Affiprep™				
0/1	18 ± 3 %	100 ± 4 %	0.023 ± 0.001 mg/mg	100 ± 4%
6.1	48 ± 5 %	267 ± 7 %	0.11 ± 0.05 mg/mg	455 ± 55%
EMPHAZE AB1				
0/1	19 ± 3 %	100 ± 2 %	0.01 ± 0.001 mg/mg	100 ± 10%
6/1	42 ± 5 %	210 ± 6 %	0.033 ± 0.005 mg/mg	321 ± 25%
CELLULOSE BEADS				
0/1	8 ± 3 %	100 ± 3 %	0.02 ± 0.003 mg/mg	100 ± 10%
211	28 ± 5 %	350 ± 7 %	0.05 ± 0.005 mg/mg	250 ± 8%
6/1	45 ± 7 %	560 ± 8 %	0.1 ± 0.008 mg/mg	500 ± 6%

a. LYSYL-MODIFIED rhPC USED AS FMA

Modified Mab was covalently coupled to the matrix by random coupling method and the coupling efficiencies of modified Mab are presented in Table II. Coupling efficiencies in the range of 99 ± 3 and 97 ± 2% were obtained for a targeted Mab density of 1.0 and 10.0 mg Mab/ml of gel, respectively.

Effect of FMA on the Antigen Binding Efficiency of Immobilized Mabs

Antigen binding efficiencies of immobilized Mab were evaluated by breakthrough analysis. The effect of modified rhPC on the efficiency of immobilized Mab on Emphaze, Affiprep, and cellulose is presented in Table III. Mab immobilized at a Mab density of 0.4 mg/ml in the absence of FMA on Affiprep has an efficiency of 18 ± 3%. Antigen binding efficiencies of 48 ± 5% were observed for Mab immobilized on Affiprep in the presence of modified rhPC at FMA:Mab ratios of 6:1 at comparable Mab densities. Mab immobilized on Emphaze in the absence of FMA gave an efficiency of 19 ± 3%. Efficiencies of 42 ± 5% were observed for Mab immobilized on Emphaze in the presence of modified rhPC at FMA:HPC4-Mab ratios of 6:1. Mab immobilized on cellulose beads in the absence of FMA gave an efficiency of 8± 3%. Efficiencies of 28 ± 5% and 45 ± 7% were observed for Mab immobilized on cellulose beads in the presence of modified rhPC at ratios of 2:1 and 6:1(FMA/Mab), respectively.

Analysis of Antigen Binding Efficiencies

Table II gives the antigen binding efficiencies $\eta_{(Ag)}$ of native and modified Mab immobilized under conventional and two-step methods. Native and/or modified Mabs when immobilized under fast reacting conditions (random coupling) gave $\eta_{(Ag)}$ of 20–26% for Mab densities of 1.0 mg/ml and 26–31% for Mab densities of 9.2 to 11.8 mg/ml, respectively. However, unmodified native Mab when immobilized with the two-step method gave $\eta_{(Ag)}$ of 23 ± 2% and 58 ± 6% at Mab densities of 1.1 and 9.3 mg/ml, respectively.

$(Fab)_2$ Determination

Tables II and III lists the total amount of $(Fab)_2$ detected per mg of immobilized Mab. Immobilizations performed, on either Affiprep or Emphaze, by masking the Mab with FMA prior to covalent coupling, gave two- to three-fold higher total amounts of $(Fab)_2$ in the pepsin digest when compared to immobilizations performed with unmasked Mab. Pepsin digestion of immunosorbents made with

unmodified and/or modified Mab did not give any significant change in the total amount of $(Fab)_2$ released. Furthermore, immunosorbents made with unmodified Mab using the two-step procedure did not give higher amounts of $(Fab)_2$ in the pepsin digests.

DISCUSSION

A loss in the functional activity of antibodies upon covalent immobilization can be attributed to local Mab density, multipoint attachment, and improper orientation [5,17]. These deleterious effects may be exacerbated by the molecular topography of the support. To gain a better understanding of the effects of supports and support activation chemistries, we have evaluated the performance of Mab immobilized on Affiprep, Emphaze, and cellulose beads in the presence of both synthetic and recombinant FMAs. In this report, results obtained with recombinant FMAs are discussed; however, similar results were obtained with synthetic masks (data not shown). Immobilizations on these supports occur via a nucleophilic attack of the primary amines on the Mab to yield a stable covalent linkage.

At low immobilized Mab densities, we postulate that orientation and multipoint attachment will have a more dominant impact on functional activity of the immunosorbent (Table IV). We have used FMAs to alleviate the effect of orientation and irreversible modification of primary amines on Mab to counteract the effect of random attachment via abundant and proximal primary amines on the Mab.

TABLE IV Ranking of factors impacting immunosorbent performance Factors impacting η_{Ag} at different regimes of immobilized antibody densities are listed and ranked. Number 1 denotes the factor having the maximum impact and number 3 denotes the factor having the minimal impact

Immobilized Mab Density	*Factors*[a]	*Rank*
LOW	Orientation	1
(< 3.0 mg Mab/ml of gel)	Multipoint Attachment	2
	Local Density	3
MODERATE	Orientation/Local Density	1
(3.0 – 6.0 mg Mab/ml of gel)	Multipoint Attachment	2
HIGH	Local Density	1
(> 6.0 mg Mab / ml of gel)	Orientation	2
	Multipoint Attachment	3

a. Please refer Velander et al., Biotech. And Bioengg., (1991).

The lower coupling efficiencies seen with immobilizations in the presence of FMAs are consistent with the physics of shielding of the Mab itself as the reactivity of the Mab is reduced. The modified rhPC possessed an avidity for Mab which was similar to that of plasma derived hPC or iinmunopurified rhPC [8]. From our previous studies we estimate that in order to attain effective shielding of the divalent Fab domains on Mab, incubations of 886-Mab with rhPC-FMA at ratios 611 were necessary [8,17]. An increase in the ratio of masked antibodies to unmasked antibodies which were immobilized may be indicated by the increase in the antigen binding efficiency from 28% to 45% for rhPC-FMA treatments of 2/1 and 611, respectively. Furthermore, no additional increase in antigen binding efficiency was observed with masked Mab at FMA/Mab ratios higher than 6/1 (data not shown). Thus, the orientation phenomena may be impacting about 50% of the immobilized antibodies for these irnmunosorbents. Our studies with synthetic masks (polymer-peptide adducts) with a molecular weight of 10K on beaded matrices, were able to impact 60% of the immobilized antibody causing an increase in antigen binding efficiency. and the increase in functional efficiency col-related well with the total amount of $(Fab)_2$ released in the pepsin digests (data not shown). The use of modified rhPC as a FMA produced similar increases in immunosorbent activity, indicating that both rhPC-FMA and synthetic 10K adduct provided sufficient and comparable shielding. FMAs which are comparable in size to the Fab domanins on the Mab (i.e. modified rhPC) may offer better Mab shielding as compared to smaller synthetic FMAs [12].

Irnmobilizations of Mab in the presence of modified rhPC gave higher total $(Fab)_2$in the pepsin digest as compared to immobilizations via random coupling. The increase in percent total $(Fab)_2$ for "FMA-masked" immunosorbents correlated well with the increases in functional efficiencies. Thus, any increase in the antigen binding efficiency of the "FMA-masked immunosorbents can be attributed to oriented immobilization as opposed to confortnational or allosteric effects.

Analysis of immunosorbents made with unmodified and/or modified Mab did not yield any significant difference in the amount of $(Fab)_2$ released. Immunosorbents made with the two-step method also gave similar amounts of $(Fab)_2$ when compared to irnmunosorbents made with reference methods. Thus, increases in the $\eta_{(Ag)}$ of iinmunosorbents made with the two-step method can be attributed to better spatial distribution of the immobilized antibody.

It is clear that "orientation" has an impact upon immunosorbent efficiency of Emphaze, Affiprep, and cellulose based immunosorbents at low antibody loadings. Multipoint attachments may still exert a deleterious effect at low antibody loadings, even with the use of FMAs. In summary, FMAs can be employed to enhance the percentage of functional antibodies relative to that obtained using

random covalent attachment. The degree of enhancement appears to be independent on the surface activation chemistry, as well as on the distribution of reactive moieties on the matrix surface at low local Mab densities. At relatively high Mab loadings, enhancement in $\eta_{(Ag)}$ can be attained by engineering a uniform installation of the ligand (i.e. Mab) by understanding the activation chemistry and the kinetics of immobilization with superimposed orientation and multipoint effects. A quantum leap in scaleable process performance can result when these effectively combine into a single matrix configuration.

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